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The overall objectives of the contract were to investigate the relationship between the activity of membrane proteins, such as protein pumps and channels, and the elastic curvature properties of the imbedding lipid bilayer. The goal was to understand if lipid composition modulates the protein activity via a coupling to the lipid monolayer elastic energies. This involved investigation both of the physical properties of lipid systems and measurement of the effects upon specific proteins. Progress on the following specific objectives are summarized:

1) To develop techniques for measuring lipid curvature elastic properties.
2) To investigate if the bilayer spontaneous curvature is regulated in bacterial cells.
3) To correlate ion-pump and channel activity with the spontaneous curvature.


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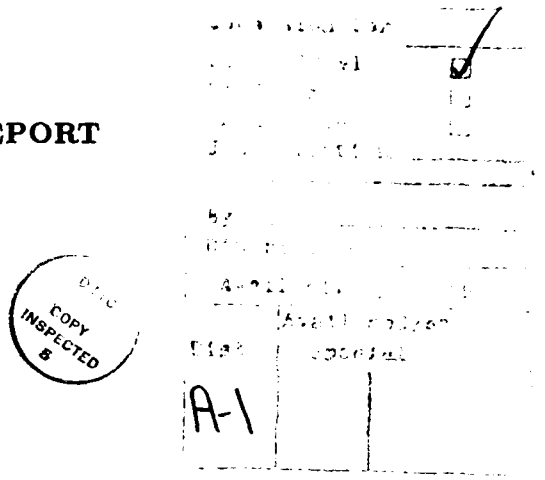

CONTRACT #N00014-86-K-0396-P-00001

PRINCIPAL INVESTIGATOR: Sol M. Gruner

INSTITUTION: Princeton University

CONTRACT TITLE: Lipid Dependent Mechanisms of Protein Pump Activity

CONTRACT PERIOD: 06/01/86 - 05/31/90

## I) Summary

The overall objectives of the contract were to investigate the relationship between the activity of membrane proteins, such as protein pumps and channels, and the elastic curvature properties of the imbedding lipid bilayer. The goal was to understand if lipid composition modulates the protein activity via a coupling to the lipid monolayer elastic energies. This involved investigation both of the physical properties of lipid systems and measurement of the effects upon specific proteins.

Specific objectives were as follows:

1) To develop techniques for measuring lipid curvature elastic properties.
2) To investigate if the bilayer spontaneous curvature is regulated in bacterial cells.
3) To correlate ion-pump and channel activity with the spontaneous curvature.

The progress achieved toward these objectives was substantial and resulted in an ONR grant to continue the work. Details of this progress follow.

## II) Membrane Elastic Properties

An understanding of the interactions of membrane proteins with lipid elastic properties depends critically on a good physical understanding of the biophysics of lipid layers. This, in turn, involves the development of the tools required to probe these properties. A multi-pronged approach was used whereby the physical properties were probed via x-ray diffraction and NMR while specimens were varied in composition, temperature, and pressure. With respect to protein function, this involved the development of methods of measuring and modulating the lipid elastic strain energies and understanding how these

strains can couple to the conformation of imbedded membrane proteins. The major accomplishments under the contract are listed below under the publications which describe the results. Full citations to the publications are listed in section (VI), below.

A) Shyamsunder et al (1988). Biochem., 27:2332-2336.

It was shown that temperature cycling of mesomorphically prone lipids can induce the formation of highly structured cubic phases with extensive long-range order. In particular, it was shown, for the first time, that a Pn3m cubic phase exists in the DOPE-water system and that this phase had not been previously observed because it was kinetically blocked. The significance of these observations were two-fold: First, it demonstrated that a huge literature on the mesomorphism of lipid phases was probably in error because certain equilibrium phases are kinetically difficult to access. Second, the cubic phase was predicted on theoretical grounds, thereby lending strong support to the curvature hypothesis (Gruner, 1989), which forms the basis for the research of this contract.

B) i) Shyamsunder et al (1989). J. Chem. Phys., 90:1293-1295.
ii) Ha & Gruner (1989). Analytical Instr. 18:197-212.

It was demonstrated that the spontaneous curvature, and hence the curvature energy, of $H_{II}$ phases was sensitively dependent on hydrostatic pressure. The magnitude of the pressure dependence was measured. It was also shown that previous reports in the literature, via FTIR, on the effects of pressure on $H_{II}$ phases were in error because of mistaken phase assignments. This paper also first reported on high pressure x-ray apparatus which was constructed to study the effects of pressure on membrane specimens. A complete characterization of the x-ray detector used for this study was presented in the Ha & Gruner paper.

C) Narayan et al (1990). Phys. Rev. A 42:7479-7482.

The determination of the structural dimensions of biomembrane phases via x-ray diffraction is usually performed by a mass assignment method which depends on the assumption that the specific volumes of water and lipid add linearly. The structural dimensions, in turn, are essential for the understanding of the curvature energy of lipid layers (Rand et al, 1990). Although the linear additivity assumption had been tested by others at low water contents, it had proven extremely difficult to directly test the validity of the assumption near full hydration, ie, precisely at the water concentration which is biologically most relevant. This paper showed that the variation of the unit cell spacing vs. pressure may be used to calculate the overall change in volume when water and lipid

are mixed at full hydration. The change in volume was shown to be less than a percent the volume of a water molecule for each water molecule transferred, thereby practically validating the use of the mass assignment technique, at least to the accuracy to which it is typically employed. The paper was important not only for the results it presented but also for the development of the technique used.

D) Gruner & Shyamsunder (1991). Ann. N.Y. Acad. Sci. (in press).

This paper explores the possible connection between the mechanism of general anaesthesia and the curvature energy of biomembranes. It was shown that the effect of alkanol anaesthetics at physiologically relevant concentrations is to decrease the spontaneous radius of curvature. Further, it was shown that the application of pressures in the range known to reverse anaesthesia are sufficient to reverse this effect. It was suggested that the change in the curvature energy alters the activity of membrane proteins, such as ion channels and pumps, which, in turn, leads to physiological anaesthesia. Although anaesthetics and pressure have long been known to modify membrane properties, and have long been implicated in the chain of events leading to anaesthesia, the measured effects on parameters such as lipid volume and fluidity were of too small a magnitude to account for anaesthesia. The significance of this paper is that it proposes, for the first time, a direct, energetically significant coupling between anaesthesia, pressure, and lipid properties at physiologically relevant magnitudes.

E) i) Turner (1990). Ph.D. Thesis.

ii) Turner & Gruner (1991). An X-ray Diffraction Reconstruction of the Inverted Hexagonal Phase in Lipid-Water Systems. (submitted to Biochem.)

iii) Turner, Gruner & Huang (1991). The Distribution of Decane Within the Unit Cell of the Inverted Hexagonal Phase of Lipid-Water-Decane Systems Using Neutron Diffraction. (submitted to Biochem.)

The lipid layer curvature vs. hydrocarbon chain packing model (Gruner, 1989), which forms the basis for the hypothesis being explored under this contract, has been subject to the criticism that there was no direct structural verification of hydrocarbon chain stress in the $H_{II}$ phase. Turner's thesis work, and the papers derived from it, sought to address this criticism. In order to do this, a novel method was developed for direct reconstruction of lipid hexagonal phase structure from the x-ray diffraction. The method was then applied to show that the interstitial regions of hexagonal phases are strained and that the strain increases dramatically as one cools to the hexagonal to lamellar phase transition

temperature. This was combined with neutron diffraction data of lipid-deuterated water-deuterated decane mixtures to prove unequivocally that decane, which is known to relax hydrocarbon packing stress, does so by preferential partitioning into the interstitial regions of the hexagonal phase, exactly as predicted by our model. The importance of these results are both in the reconstruction procedures which were developed and in the results which were found. The results lend strong support to the hypothesis of lipid curvature strain in biomembrane bilayers.

F) i) So (1991) Ph.D. thesis (expected in the Fall of 1991).
ii) So, Shyamsunder & Gruner (1991) (in preparation).

The Ph.D. work of Peter So, which is now in its final stages, is a detailed structural and thermodynamic investigation of the interaction of hydrostatic pressure and lipid membrane curvature. A high pressure x-ray apparatus was constructed and used to collect high pressure diffraction data on lipid-water systems from 1 to 3 kbar. The direct reconstruction method of Turner (1990) was then used to show how pressure affects the density of headgroups and hydrocarbon tails in membranes and how this couples to the spontaneous curvature. High pressure dilatometry was then performed, using novel dilatometric apparatus constructed for this purpose, to measure the volume changes which occur vs. pressure. This is difficult because the volume changes, especially across phase transition boundries, are very small. All of the x-ray data, and most of the dilatometric data has been collected. The plan is to use the Clausius-Clapeyron relation, in combination with the x-ray structural data, to extract the pressure induced changes in the thermodynamic heats of transition.

G) Gruner (1989). Rev. Sci. Instr. 60:1545-1551.

The area x-ray detectors used for the studies performed under this contract were described. These detectors are presently the most advanced CCD-based area detectors available and are of great interest for many different x-ray diffraction problems.

H) i) Gruner (1989). J. Phys. Chem. 93:7562-7570.
ii) Tate et al (1991). Chem. Phys. Lipids 57:147-164.
iii) Gruner (1991). Lipid Membrane Curvature Elasticity and Protein Function, in *Biologically Inspired Physics*, Nato Advanced Study Series, L. Peliti & S. Leibler, eds. (Plenum Press, N.Y., in press).
iv) Gruner (1991). Coupling Between Bilayer Curvature Elasticity and Membrane Protein Activity, in *Biomembrane Electrochemistry*, M.Blank & I. Vodanoy, eds. (ACS Books, Washington, D.C., in press).

v) Gruner (1991). Nonlamellar Lipid Phases, in *The Structure of Biological Membranes*, P. Yeagle, ed. (CRC Press, Boca Raton, FL, in press).

These are all invited papers which review the work on lipid phase behavior and the coupling to membrane proteins. The papers effectively are published summaries of the work carried out under this contract.

### III) Membrane Protein Reconstitution Studies

Extensive work was performed on attempting to correlate the activity of the $Ca^{++}$ ATPase of sarcoplasmic reticulum to the spontaneous curvature when reconstituted in lipid mixtures of defined composition. The experiments were predicated on a series of literature reports which qualitatively correlated the activity of the protein pump with the hexagonal-phase forming tendencies of various lipid mixtures (Navarro et al, 1984; Cheng et al, 1986; Cheng & Hui, 1986). The goal was to repeat the experiments using lipids of well-defined composition for which the curvature energies could be quantitatively measured via x-ray diffraction using the techniques developed under this contract and those described in Rand et al (1990).

Initial experiments yielded data in qualitative agreement with the literature reports and demonstrated a correlation between the activity of the pump and the spontaneous curvature of the membrane. Specifically, the activity of the pump was determined by measuring the ratio of the rate of ATP hydrolysis to the rate of radio-calcium sequestration into lipid vesicles with reconstituted pumps. The activity was plotted vs. a parameter relating to the curvature energy of the lipid bilayers. Upon review of the results for publication, it was pointed out that the data did not exclude the possibility of systematic variations in calcium leakage from the vesicles which might also correlate with the spontaneous curvature of the bilayers. If such an effect did occur, then the ATPase activity assay would be ambiguous because the rate of $Ca^{++}$ sequestration would be due both to the rate at which $Ca^{++}$ is pumped by the protein and the rate at which it leaks out of the vesicles.

A series of control experiments with pure lipid vesicles failed to show that $Ca^{++}$ leakage correlated with the lipid composition. However, after much experimentation, it was shown that there was a systematic correlation of $Ca^{++}$ leakage if the vesicles contained the ATPase, even if the protein was not functioning. This was shown by reconstituting proteoliposomes with encapsulated $Ca^{++}$, but in the absence of ATP. Apparently, in the presence of the protein, there is a lipid dependent ionic leakage which correlates with the hexagonal phase forming tendencies of the lipid. This was a great disappointment because it meant that the classic ATPase assay (and, incidentally, a great deal of literature based

on the assay) was flawed and could not be used in situations in which the lipid composition was varied. The experiments were terminated for lack of a suitable assay of the activity of the protein.

It is important to recognize that these results, although disappointing, do not provide evidence either for or against the hypothesis explored under this contract. Rather, the experiments showed that the $Ca^{++}$ ATPase assay is unsuitable for probing correlations between lipid composition and the activity of the protein. In consequence, we have undertaken a search for an alternative membrane protein system which is not plagued by assay problems having to do with ionic leakage. In this regard, it of interest to note that recent experiments on the opening and closing of the calcium-activated potassium channel appear to have lipid dependent effects which appear promising (Chang et al, 1991).

## IV) Bioregulation of Curvature Strain in Bacterial Membranes

The idea behind these experiments is that if lipid curvature strain is important toward membrane protein function, then the strain is likely to be carefully regulated in cell membranes. The experimental protocol was to obtain lipid extracts from bacteria grown under nutrient conditions which result in changes in the biomembrane lipid composition. Since many different compositions may be selected which yield any given value of the spontaneous curvature, it would be of interest to see if the actual compositions were consistent with a restricted range of variation of the curvature. A restricted range of curvature variation in the face of a large range of variation in composition would argue in favor of bioregulation of the spontaneous curvature.

The difficulty of these experiments is that one must obtain a pure fraction of a single membrane from an organism for which the membrane lipid composition can be readily manipulated. Lindblom et al (1986) published a study which suggested that Acholeplasma llaidlawi A might be a suitable organism for three reasons: (1) It has only a single membrane. (2) The presence of exogenous fatty acid leads to composition variations of the membrane. (3) The Lindblom et al (1986) study suggested that the transition temperatures from lamellar to nonlamellar phases were being held approximately constant in the face of substantial lipid composition variation. The latter feature is important because our work has shown that for lipids with a restricted set of chain lengths, the transition temperature correlates with the spontaneous curvature (see Gruner, 1989).

The llaidlawi A strain is difficult to grow. The Lindblom group has the most experience with growing and manipulating this organism. Accordingly, arrangements were made with the Lindblom group to grow the organisms under appropriately defined conditions

and deliver the lipid extracts for diffraction measurements of the spontaneous curvature. The work was delayed by difficulties in obtaining good growth and suitable amounts of the extracted membranes. However, the Lindblom group finally succeeded in delivering adequate amounts of material during January of 1991. Experiments on the membrane extracts are now in progress.

## V) References

Auxillary literature referenced above, but not listed in the publications section (VI), below, are given:


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## VI) Publications, Theses and Abstracts


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Gruner, S.M. (1990). Relations between curvature elasticity, nonlamellar phases, and biomembrane function. Biophys. J. 57: 20a.

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